1 Co-spray dried resveratrol and budesonide inhalation formulation for reducing

2 inflammation and oxidative stress in rat alveolar macrophages

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Abstract

Oxidative stress is instrumental in the pathogenesis and progression of chronic obstructive pulmonary disease (COPD). Novel therapeutic strategies that target macrophages, based on the use of antioxidant compounds, could be explored to improve corticosteroids responses in COPD patients. In this study, inhalable microparticles containing budesonide (BD) and resveratrol (RES) were prepared and characterized. This approach was undertaken to develop a multi-drug inhalable formulation with anti-oxidant and anti-inflammatory activities for treatment of chronic lung diseases. The inhalable microparticles containing different ratio of BD and RES were prepared by spray drying. The physico-chemical properties of the formulations were characterized in terms of surface morphology, particle size, physical and thermal stability. Additionally, *in vitro* aerosol performances of these formulations were evaluated with the multi-stage liquid impinger (MSLI) at 60 and 90 l/min, respectively. The cytotoxicity effect of the formulations was evaluated using rat alveolar macrophages. The biological responses of alveolar macrophages in terms of cytokine expressions, nitric oxide (NO) production and free radical scavenging activities were also tested. The co-spray dried (Co-SD) microparticles of all formulations exhibited morphologies appropriate for inhalation administration. Analysis of the deposition profiles showed an increase in aerosol performance proportional to BD concentration. Cell viability assay demonstrated that alveolar macrophages could tolerate a wide range of RES and BD concentrations. In addition, RES and BD were able to decrease the levels of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in lipopolysaccharide (LPS) induced alveolar macrophages.

48   This study has successfully established the manufacture of Co-SD formulations of RES

49   and BD with morphology and aerosol properties suitable for inhalation drug delivery,

50   negligible *in vitro* toxicity and enhanced efficacy to control inflammation and oxidative

51   stress in LPS-induced alveolar macrophages.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) represents one of the leading causes of morbidity and mortality worldwide (Viegi, Scognamiglio et al. 2001). COPD is a lung disease characterized by chronic inflammation, airflow limitation, hyper mucous production, emphysema, bronchoconstriction, a decline of respiratory activity and eventual death (Barnes 2007). The pathogenesis of COPD is multi-factorial which includes genetic predisposition, age, inhaled pollution and cigarette smoke. Previous studies have shown that cigarette smoke (CS) is the main risk factor for the development and progression of COPD (Rabe, Hurd et al. 2007). This is because CS causes a production of reactive oxygen species (ROS) that increase the oxidative stress and for this reason it is implicated in the pathogenesis and in irreversible airway inflammation. Oxidative stress causes airway inflammation by stimulating the release of inflammatory mediators such as IL-6, IL-8 and TNF-α. These inflammatory mediators result in an increase of ROS and hence an increase in oxidative stress in the lungs (Rahman and Adcock 2006). Furthermore, COPD exacerbations caused by chronic bacterial infection can result in additional airway inflammation owing to the further release of pro-inflammatory mediators (Khair, Davies et al. 1996).

Alveolar macrophages are one of the first lines of defence of the respiratory tract against inhaled noxious agents. Although airway epithelial cells as a whole are involved in COPD, several studies have demonstrated that alveolar macrophages play an important role in the pathogenesis of the disease (Tetley 2002; Hodge, Hodge et al. 2007), mainly in smokers, by regulating the release of inflammatory mediators that attract neutrophils into the airway (Kent, Smyth et al. 2008). Corticosteroid molecules are able to suppress

96 the release of these inflammatory mediators in alveolar macrophages but these drugs are

97 relatively ineffective in COPD patients (Barnes, Ito et al. 2004, Bhavsar, Hew et al.

98 2008). For this reason a novel therapeutic strategy is needed.

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100 The current first-line therapy for COPD involves the use of bronchodilators such as long

101 acting beta agonists (LABA) in combination with inhaled corticosteroids. However,

102 unlike other inflammation-based diseases, such as asthma, corticosteroids are less

103 effective in improving lung function of COPD patients, and have limited effect in

104 reversing the progression of tissue damage (Pauwels, Löfdahl et al. 1999; Pauwels,

105 Buist et al. 2001; Dahl, Chung et al. 2010; Vogelmeier, Hederer et al. 2011).

106 Furthermore, previous studies have shown that ROS have been implicated in initiating

107 inflammatory responses in the lungs through the activation of transcription factors such

108 as nuclear factor-kappa B (NF-κB) (Rahman and MacNee 1998). This results in vicious

109 cycle of oxidative stress by ROS and airway inflammation. Histone deacetylases

110 activities are required for NF-κB blockade by corticosteroid receptors (Barnes, Ito et al.

111 2004; Barnes 2009). In several cases COPD patients became non-responsive to

112 corticosteroid treatment as histone deacetylases (HDAC$_2$) activities can become

113 inhibited in presence of oxidative stress (Barnes, Ito et al. 2004).

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115 For these reasons, the use of anti-oxidant compounds in association with one of the

116 corticosteroids drugs could provide a new therapeutic approach for the treatment and

117 management of COPD.

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Polyphenolic compounds are potential candidate molecules since these compounds naturally exhibit potent anti-oxidant and anti-inflammatory activities (Biswas, Hwang et al. 2013). Resveratrol (3, 5, 4- trihydroxystilbene) (RES) is a naturally occurring polyphenolic compound found in a large number of plant species (e.g. grapes, berries and legumes) and in red wine. Resveratrol is a light sensitive molecule with two isoforms, *cis*-resveratrol and *trans*-resveratrol, the trans form being more stable and also the more biologically activity form (Neves, Lucio et al. 2012). The anti-oxidant activity of this polyphenol is due to its ability to scavenge free radicals (Arts and Hollman 2005). Furthermore, different studies have shown the differential properties of resveratrol as anti-inflammatory, anti-allergic, antiviral, anti-carcinogenic and anti-asthmatic (Cheong, Ryu et al. 1999; Docherty, Fu et al. 1999; Manna, Mukhopadhyay et al. 2000; Alarcón de la Lastra and Villegas 2005; Faith, Sweet et al. 2006; Athar, Back et al. 2009; Lee, Kim et al. 2009). Specifically in the lungs, *in vitro* and *in vivo* experiments have shown that RES can reduce inflammation in lung cells, scavenging oxygen-derived free radicals; subsequently, RES maybe a potential adjunct therapy in the treatment of COPD (Trotta, Lee et al. 2015). In addition, RES has been shown to inhibit the release of inflammatory cytokines from alveolar macrophages in COPD and therefore can be considered a suitable candidate for pharmacotherapy of macrophages (Culpitt, Rogers et al. 2003).

The aim of this study was to develop inhalable microparticles containing RES and budesonide (BD), a common anti-inflammatory corticosteroid. To the authors' knowledge, this is the first attempt to deliver a combination formulation containing anti-oxidant and anti-inflammatory compounds for improvement of COPD. Different series

of co-spray dried (Co-SD) formulations were prepared and the physico-chemical characteristics and *in vitro* aerosol performance were investigated. Importantly, the biological responses of alveolar macrophages cell lines in terms of cell viability, anti-inflammatory and anti-oxidant activities were evaluated with the prepared spray dried formulations.

2. Experimental methods

2.1. Materials

Resveratrol, *trans*-3,4',5-trihydroxystilbene, (RES) was purchased from Fagron Italia (Bologna, Italy). Budesonide (BD) used in this study was supplied by Yicheng Chemical Corp, Jiangsu, China. Nitro-L-arginine methyl ester (L-NAME), α-Lipoic acid, L-ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipopolysaccharide (LPS) from *Escherichia coli* and 2,3-diaminonaphthalene (DAN) were purchased from Sigma-Aldrich (Sydney, Australia). Other cell culture reagents inculding phosphate buffer saline (PBS), fetal bovine serum (FBS) and Ham's F-12 nutrient mix media were purchased from Invitrogen, (Sydney, Australia). Elisa kit for determination of inflammation markers such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were supplied by BD Bioscience (Sydney, Australia). All solvents used were analytical grade and purchased from Biolab (Victoria, Australia).

2.2. Preparation of spray dried (SD) microparticles

Single and combination microparticles were produced by spray drying RES and/or BD using a Buchi B-290 Mini spray dryer (Buchi, Switzerland) under conditions listed in Table 1. Both RES and BD, either alone or in combination, were dissolved in ethanol-

167   water (80:20% v/v) and spray dried using a nozzle of 1.4 mm at feed rate of 40% and

168   aspiration of 100% in a close loop configuration. Single or combinations of RES and

169   BD with final dry weight percentages (%w/w) were labelled as follow: 100% RES,

170   75%:25% RES-BD, 50%:50% RES-BD, 25%:75% RES-BD and 100% BD.

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172   *2.3. Physico-chemical characterization of SD formulations*

173   *2.3.1. Scanning electron microscopy (SEM)*

174   Scanning electron microscopy was used to study the morphology of the SD

175   formulations. Briefly, SD-RES, SD-BD and Co-SD RES-BD formulations were

176   dispersed on carbon tapes, placed onto aluminium stubs and coated with gold at 15 nm

177   thickness (JEOL USA Smart Coater). A bench top SEM (JMC, 6000 JEOL, Japan)

178   operating at 15KV was used for imaging samples.

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180   *2.3.2. Laser diffractic of Co-SD microparticles*

181   Particle size distribution of Co-SD microparticles were determinate by laser diffraction

182   (Mastersizer 3000, Malvern, Worcestershire, United Kingdom). Approximately 10 mg

183   of microparticles were dispersed in air with a feed pressure and feed rate of 4 bars and

184   35%, respectively. Co-SD formulations   were analysed in triplicate with an obscuration

185   value between 0% and 15%. Moreover, a refractive index of 1.67 was used for all

186   measurements and was calculated by the average of the refractive index of the two

187   single components (Salama, Young et al. 2014, Trotta, Lee et al. 2015).

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189   *2.3.3. Thermal analysis of SD formulations*

190 The thermal responses of the raw RES and BD and the single and Co-SD formulations

191 were investigated using differential scanning calorimetry (DSC; DSC823e, Mettler-

192 Tolledo, Switzerland). Approximately 3 to 5 mg of powder were weighed and crimp-

193 sealed in DSC aluminium pans and heated at 10°C/min over a temperature range of 25–

194 320 °C. The exothermic and endothermic responses of the SD microparticles, raw RES

195 and raw BD were determined using STARe software V.11.0x (MettlerToledo). In

196 addition, the temperature stability and solvent evaporation of each formulation was

197 assessed using the thermal gravimetric analysis (TGA; Mettler-Toledo, Switzerland).

198 Approximately 13 mg of SD powders were placed onto aluminium crubicle pans. The

199 weight loss of the samples was evaluated by heating the samples from 25–200°C with

200 scanning rate of 10ºC /min under constant nitrogen gas at flow 110 ml/min. Data were

201 analysed using STARe software V.11.0x (MettlerToledo, Switzerland) and expressed as

202 the percentage of weight loss with respect to initial sample weight.

203

204 *2.3.4. In vitro aerosol performance*

205 The multi stage liquid impinger (MSLI; CopleyScientific Ltd., Nottingham, UK) was

206 used to evaluate the aerosol performance of SD microparticles, as per the methodology

207 outlined in the British Pharmacopiea(Pharmacopoeia 2009). Approximately 10 mg of

208 SD powders was loaded in a size 3 gelatin capsules (Capsugel®, Sydney, Australia) and

209 placed into the dosage chamber of standard-resistance RS01 dry powder inhalation

210 device (Plastiape®, Osnago, Italy). The device was connect to a custom-built

211 mouthpiece addpator connected to a US Pharmacopeia induction port (throat),

212 connected to the MSLI. The aerosol performance was assessed at two different flow

213 rates, 60 l/min and 90 l/min (calibrated using a suitable flow meter). After actuation, the

214 device, capsule, adaptor, throat, all MSLI stages and filter were washed separately with

215 methanol:water (80:20 %v/v) into suitable volumetric flasks. The samples were

216 analysed using high performance liquid chromatography (HPLC). All experiments were

217 conducted in triplicate and the aerosolization efficiency was evaluated in terms of fine

218 particle dose (FPD) (amount of RES and BD recovered from stage 3 to filter,

219 corresponding to the amount of microparticles with a diameter < 6.8 µm), fine particle

220 fraction of the loaded dose (FPF$_{LD}$) (percentage ratio of FPD respect the total amount of

221 RES and BD collected from device, capsule, adaptor, throat and all MSLI stages and

222 filter).

223

224 *2.4. HPLC quantification of RES and BD*

225 Resveratol and BD were quantified using a validated HPLC method. A Shimadzu

226 Prominence UFLC system equipped with a DGU-20 A5R Prominent degasser unit, LC-

227 20 AD Liquid chromatography, SIL-20A HT Autosampler and SPD-20A UV-Vis

228 detector was used (all Shimadzu Corporation, Japan). For both, RES and BD, the

229 volume of sample injection was 100 μl and the detection wavelength for BD and RES

230 were 243 nm and 306 nm, respectively. Quantification of BD was conducted using

231 methanol:water 80:20 (%v/v) as the mobile phase at flow rate of 1 ml/min in isocratic

232 mode with a Luna C18 column (3 μm, 4.6 × 150 mm) (Phenomenex, Sydney,

233 Australia). The linearity was confirmed between 0.2 and 50 μg/ml (R^2 = 0.999). For

234 quantification of RES, the mobile phase consisted of methanol:water 60:40 (%v/v) with

235 0.5% acetic acid (%v/v). Samples were analysed using a XbridgeTM column (5 μm, 4.6

236 × 150 mm), (Waters, Massachusetts, USA) at a flow rate of 0.7 ml/min. The content of

237   resveratrol was quantified from the peak area correlated with the predetermined

238   standard curve between 0.2 and 50 μg/ml (R^2 =0.999).

239

240   *2.5. In vitro biological responses*

241   *2.5.1. Alveolar macrophage NR8383 cell line and culture conditions*

242   Rat alveolar macrophage NR8383 cells were obtained from the American Type Culture

243   Collection (ATCC, Manassas, VA, USA). Cell lines were tested for mycoplasma

244   contamination (mycoplasma PCR detection Kit) and found to be negative. Cells were

245   maintained in complete Ham's F-12 nutrient mix medium with 10% (v/v) heat-

246   inactivated FBS in a humidified atmosphere containing 5% CO_2 at 37 °C. Medium was

247   changed every 3 days and passaged (passage number 20-30) according to ATCC

248   recommended guidelines.

249

250   *2.5.2. MTS cell cytotoxicity assay in response to SD formulations*

251   To evaluate the suitability of delivering RES and BD into lungs, a *in vitro* MTS

252   cytotoxicity assay was conducted. Alveolar NR8383 macrophages were seeded onto 96-

253   well plates at a density of 50,000 cells/well and incubated at 37°C in a humidified

254   atmosphere at 5% CO_2 overnight. This was followed by the addition of drugs (RES and

255   BD) with increasing concentrations, from 0.612 μM to 50 μM, either as single or

256   combined formulations. After 72 h of incubation, 20 µl of CellTiter 96® Aqueous assay

257   (MTS reagent, Promega, USA) was added into each well and incubated for 4 h. The

258   absorbance of the samples were measured at 490 nm with microplate reader (Wallac

259   1420 VICTOR^{2TM}, Multilaber Counter, Massachusetts, USA). Experiments were

260 performed in triplicate and data expressed as % cell viability relative to untreated

261 control.

262

263 *2.6. In vitro anti-oxidant activities of SD formulations*

264 *2.6.1. DPPH radical scavenging activity*

265 The anti-oxidant activity of the SD formulations were evaluated using a DPPH (2,2-

266 diphenyl-1-picrylhydrazyl) assay according the method described by Basnet *et al*.

267 (Basnet, Hussain et al. 2012). Different concentrations (1 µM to 1 mM) of RES and BD

268 either in single or combination formulation were reacted with 60 µM DPPH ethanolic

269 solution. After 30 min of incubation in the dark, the absorbance of the reactive mixture

270 was measured at 520 nm. The same concentrations of Nitro-L-arginine methyl ester (L-

271 NAME) and α-Lipoic acid as well as ascorbic acid were used as negative and positive

272 controls, respectively. All experiments were conducted in triplicate.

273

274 *2.6.2. NO production using 2,3-diaminonaphthalene (DAN) assay*

275 The ability of SD formulations to reduce the production of nitric oxide (NO) in LPS-

276 induced alveolar macrophages were evaluated using DAN assay in accordance to the

277 method described previously (Choi, Zhang et al. 2009, Trotta, Lee et al. 2015). Briefly,

278 alveolar macrophage cells were treated with 5 ng/ml LPS for 24 h. This was followed

279 by treatment with SD formulations with different concentrations. Samples were

280 collected at different time points (12 h to 72 h). Ascorbic acid and L-NAME were used

281 as positive and negative control, respectively. Serial concentrations of nitrite (0.19 to 25

282 µM) were prepared as standard. The concentration of NO was determined by measuring

283 the fluorescence intensity (excitation=360 nm and emission= 430 nm).

284

285 2.7. Anti-inflammatory activity of SD formulations against LPS-induced alveolar

286 macrophages NR8383 cells

287 The anti-inflammatory effects of the SD formulations were evaluated using a LPS-

288 induced alveolar macrophages cell study. Approximately 500,000 cells/well were

289 seeded into 6 well plates with 5 ng/ml of LPS before incubation for 24 h at 37°C, with

290 5% CO_2. The cells were subsequently treated with the drugs at different concentrations,

291 ranging from 3.125 μM to 50 μM, at different incubation time points. It should be noted

292 that the LPS concentration used in this experiment was found not toxic to the cell (cell

293 viability above 95%) as the cell viabilty was pre-determined before the study. At

294 different time points, the samples were harvested by centrifugation at 13,000 rpm at 4°C

295 for 5 min. A clear supernatant was kept in –80°C. Cytokine expression (IL-6 and TNF-

296 α) was measured using Rat IL-6 and TNF-α ELISA kits according to the manufacturer

297 instructions.

298

299 *2.8. Statistical analysis*

300 One-way ANOVA or unpaired 2-tailed t-tests were performed to determine the

301 significance (which was quoted at the level of $p < 0.05$) between treatment groups and

302 control.

303

304 **3. Results and discussion**

305

306 *3.1. Physical-chemical characterization of SD formulations*

307 *3.1.1. Assessment of particle size and morphology*

308   In this study, inhalable microparticles containing anti-oxidant RES and anti-

309   inflammatory BD compounds were formulated using SD. Particle sizes with uniform

310   distributions within inhalable range were obtained via manipulation of the spray-drying

311   parameters, which included inlet temperature, outlet temperature, atomisation, and feed

312   rate of solution. Additional factors such as ratios of co-solvent and concentrations feed

313   solutions led to significant changes in particle distribution as well as yield. The inlet

314   temperature range was chosen so that the outlet temperature would be below the glass

315   transition temperature (T_g) of BD and RES, which are reported as ~90°C and > 340 °C,

316   respectively (Nolan, Tajber et al. 2009; Cash, Davis et al. 2013). Through the

317   optimization of spray-drying conditions, five formulations with RES to BD

318   concentration were successfully prepared. These included: 100% BD,

319   75%BD/25%RES, 50%BD/50%RES, 25%BD/75%RES and 100% RES. Throughout

320   the manuscript, these formulations were denoted as formulation 0%, 25%, 50%, 75%

321   and 100% respectively, in accordance to the percentage w/w RES in the final SD

322   particles.

323

324   As seen from the SEM images presented in Figure 1, the microparticles of single spray

325   dried and Co-SD combinations at different concentrations showed spherical geometries;

326   morphological characteristic typical of a spray-drying process. Qualitative measurement

327   indicated that the particles were between 1 to 5 μm in size. Uniform spherical

328   microparticles with smooth surface morphology were characteristics of SD BD alone

329   (Figure 1A). In comparison, spray dried RES alone (100%) exhibited spherical particles

330   with rough surfaces (Figure 1E). When Co-SD, the particles exhibited a change in

331   morphology between the two single drug formulations based on the ratio of BD to RES.

332   Interestingly, overall Co-SD microparticles exhibited increase in smoothness of

333   particles' surface proportional to the increase of BD concentration (Figure 1B, C and

334   D). It is speculated that, as BD is more hydrophobic and with higher molecular mass, in

335   the binary system it would preferentially accumulate on the air-liquid interface of the

336   droplet during the spray drying process (Parlati, Colombo et al. 2009).

337   Particle size analysis of Co-SD microparticles were measured using laser diffraction.

338   The volume weighted median diameter (D_{50}) of SD BD was 1.0 ± 0.01 µm, significantly

339   smaller ($p < 0.05$) than SD RES (7.74 ± 1.66 µm). A gradual increase in particle size for

340   Co-SD formulations with increasing RES concentration was observed. As such, the

341   diameter for Co-SD containing 25%, 50% and 75% RES were 1.2 ± 0.02, 2.32 ± 1.01

342   and 6.23 ± 1.32 µm, respectively. Moreover the presence of BD on the surface reduced

343   the cohesiveness between particles of RES, thus resulting in decreased particles'

344   agglomeration and increased aerosol performance, as reported below (Adi, Adi et al.

345   2008).

346

347   *3.1.2 Thermal characteristics of spray dried formulations*

348   The thermal response of spray dried formulations containing either RES and/or BD is

349   shown in Figure 2. Both thermograms of crystalline raw BD and RES demonstrated

350   only a single endothermic peak indicative of melting at 260°C and 270°C, respectively.

351   Spray drying processes did not change RES crystallinity, as seen in the presence of

352   single endothermic melting at 269°C for SD RES alone (Trotta, Lee et al. 2015). The

353   DSC thermogram for SD BD alone however showed a broad exothermic peak at 130°C,

354   followed by a sharp endothermic peak at 260°C. The presence of exothermic peak prior

355   to melting suggests that SD BD particles were amorphous and underwent phase

transition from amorphous to crystalline when heated to 130°C. In general, the melting

peaks of Co-SD formulations were shifted to lower temperature. In addition,

irrespective of the concentrations of RES and BD used, the Co-SD formulations

exhibited a 'new' endothermic peak at 190°C, which was not thermodynamically

related to raw RES or BD compounds (Figure 2). The presence of this peak suggested

the chemical interaction between the two compounds which resulted in the shifting of

the melting peak proportional to the strong solid–solid interaction (Budavári, Zelko et

al. 1999) For instance, for Co-SD containing 75% RES, two endothermic peaks were

observed at 190°C and 257°C, respectively. In the Co-SD formulations containing 75%

BD, an exothermic peak at 167 °C and one endothermic peak at 206 °C were also

observed.

Further, spray dried formulations were stable up to 200 °C with TGA data reporting a

weigh lost ≤ 0.1% w/w.

3.2. Aerosolization efficiency of spray dried formulations

The evaluation of aerosol efficiency for dry powder inhalers is an important tool for

predicting the amount of the inhalable microparticles that could reach the lungs. The

aerosol performance of single SD and the Co-SD formulations were tested using the

MSLI at two different flow rates (60 l/min and 90 l/min). Data are expressed as the

percentage deposition of BD and RES recovered in each stage of MSLI, throat, and

device over the total mass calculated (Figure 3 and 4). The FPF_{LD} values of SD BD

were significantly higher than for SD RES at both flow rates ($p < 0.001$). At 60 l/min,

the FPF for SD BD and SD RES were 39.4 ± 2.8% and 26.3 ± 1.5%, respectively.

Meanwhile, the FPF of SD BD (45.7 ± 1.4%) was 2-fold higher than SD RES (25.8 ±

380 4.1%) when the flow rate was set at 90 l/min. Furthermore, SD RES had a higher throat

381 deposition (11.5 ± 1.6% and 17.8 ± 0.9%) compared to SD BD with values of

382 deposition of 3.6 ± 0.9% and 5.0 ± 0.3% at 60 l/min and 90 l/min, respectively.   This

383 could be attributed to the adhesive/cohesive nature of RES and high surface area of the

384 microparticles, which promoted agglomerates; hence the poor dispersion at set flow

385 rates (Figure 3 and 4). It was also observed that deposited doses of each component

386 (RES and BD) in the throat, devices and all stages of the MSLI had no significant

387 differences, irrespective of the Co-SD combinations. These results indicate that the Co-

388 SD particles were homogeneous in composition, rather than binary system containing

389 individual components. Analysis of the deposition profiles of Co-SD formulations

390 showed an increase in aerosol performance proportional to RES decreasing

391 concentration. The FPF of RES for Co-SD formulations containing 25%, 50% and 75%

392 RES were 42.5 ± 1.7%, 38.8 ± 2.9%, and 23.8 ± 3.7%, respectively at 60 l/min (Figure

393 3). Similarly, the FPF calculated for BD were 42.5 ± 1.6%, 38.5 ± 3.1%, and 23.0 ±

394 1.2%, respectively (Figure 3). At 90 l/min, the FPF of RES for Co-SD formulations

395 containing 25%, 50% and 75% RES were 46.7 ± 2.8%, 41.7 ± 4.4%, and 25.9 ± 4.2%,

396 respectively (Figure 4). Moreover the FPF of BD for Co-SD formulations containing

397 25%, 50% and 75% RES were 46.5 ± 2.8%, 41.2 ± 4.3% and 26.4 ± 2.7% at 90 l/min.

398

399 *3.3. In vitro activity of RES and BD using alveolar macrophages cells*

400 *3.3.1. Viability of alveolar macrophages cells to RES and BD*

401 It is widely accepted that inflammation and oxidative stress is the central pathogenesis

402 contributing to the development of chronic lung diseases such as asthma and COPD

403 (MacNee 2001). Continuous exposure to noxious agents such as cigarette smoke,

404 viruses, bacteria and foreign particulates promotes inflammation and oxidative stress in

405 lung cells and alveolar macrophages. Budesonide is the treatment of choice to control

406 inflammation but some COPD patients are unresponsive towards corticosteroids.

407 Therefore, combining BD with RES, which possesses strong anti-oxidant and anti-

408 inflammatory properties, could be beneficial for COPD treatment. Prior to anti-

409 inflammatory and anti-oxidant studies, any potential cytotoxicity effects of RES and BD

410 towards alveolar macrophage cells need to be evaluated. Different SD formulations,

411 both as combination or single compounds, were added to rat alveolar macrophages with

412 increasing concentrations (0.612 to 50 μM) and evaluated using the MTS cytotoxicity

413 assays and the data are shown in Figure 5. In general, the viability of alveolar

414 macrophage cells did not decrease significantly with RES and/or BD increase regardless

415 of in single (Fig. 5 A) or combination formulations (Fig.5 B). As shown in Figure 5A,

416 alveolar macrophage cells demonstrated satisfactory tolerance towards RES and BD in

417 the range of concentrations investigated. These results are in accordance with published

418 studies indicating that RES was well tolerated by airway lung cells, such as Calu-3,

419 A549 and murine macrophages cells (Billack, Radkar et al. 2008; Liu, Tsai et al. 2010;

420 Trotta, Lee et al. 2015). Moreover BD has been shown to be relatively safe for alveolar

421 macrophages even at micromolar concentration (Zetterlund, Larsson et al. 1998).

422

423 *3.3.2. Anti-oxidant activities of Co-SD formulations*

424 The anti-oxidant activity of RES and BD were investigated by measuring their ability to

425 scavenge free reactive radicals, using the DPPH assay (Figure 6A). The hydrogen

426 donating molecules reduce the free radical DPPH by pairing its odd electron to

427 hydrogen, thus causing a colour change from purple to yellow (a decrease in

428 absorbance). Lipoic acid and L-NAME were used as negative controls while vitamin C,

429 a potent free radical scavenger, was used as positive control in this study (Figure 6A).

430 As expected, no scavenging activities were observed for lipoic acid and L-NAME even

431 at high concentration (500 µM). Vitamin C demonstrated the highest anti-oxidant

432 potential by reducing up to 80% DPPH free radicals at 40 µM which was consistent

433 with previous study (Trotta, Lee et al. 2015). As for BD, no detectable radical

434 scavenging activities were found in the ranges of concentrations studied (25 to 500

435 µM). Meanwhile, it was observed that the anti-oxidant activities of RES were dose-

436 dependent. The increase of RES from 2 to 500 µM led to significant reduction of free

437 DPPH radicals, whereby only 87.8% and 25.2% of DPPH remained, respectively

438 (Figure 6A). The dose-response curve of RES showed a gradual increase in DPPH

439 scavenging activity while dramatic elevation of anti-oxidant activity was observed for

440 vitamin C. For instance, approximately 70% and 31% of DPPH free radicals remained

441 after treatment with 3.9 and 7.8 µM vitamin C, respectively (Figure 6A).

442

443 Oxidative damage in lung tissues caused by stimuli such as cigarette smoke is well

444 known. The introduction of an anti-oxidant molecule such as resveratrol to existing

445 therapies to potentially treat oxidative-stress related lung injury (i.e. COPD) could

446 consequently be advantageous. In both smokers and COPD patients it was shown that

447 the expression of glutamate-cysteine ligase (GCL) and glutathione (GSH) was reduced

448 compared to healthy subjects, hence an indication of lung injury caused by oxidative

449 stress (Harju, Kaarteenaho-Wiik et al. 2002; Cerqueira, Khaper et al. 2013), while

450 resveratrol can stimulate lung repair by enhancing GCL and GSH productions (Kode,

451 Rajendrasozhan et al. 2008).

452

Steroid resistance in COPD patients has been linked to nitrosative stress which is contributed by NO production and protein nitration in airway cells (Barnes, Ito et al. 2004). In this study, the inhibitory effect of spray dried formulations on NO production was investigated using LPS-induced alveolar macrophage cells (Figure 6B and 6C). It has been demonstrated that LPS stimulated iNOS expression in macrophages cells, which in turn led to enhanced production of NO via conversion of L-arginine to L-citrulline (Jøraholmen, Škalko-Basnet et al. 2015). When high-output of NO levels is non-attenuated, it could facilitate synthesis of peroxynitrite intermediates, which react to form 3-nitrotyrosine that causes severe lung epithelial damage (Peng, Abdulnour et al. 2005, Crosswhite and Sun 2010). In a study by Peng et al, the increase of NO production was correlated to 3-nitrotyrosine concentration in lungs (Peng, Abdulnour et al. 2005). It is therefore likely that during COPD exacerbation caused by bacterial infection, activated alveolar macrophages resulting in elevated NO levels and inflammatory cytokines, which lead to lung injury and fibrosis. These unstable NO radicals are rapidly converted to NO_2^- or NO_3^-.

Therefore the amount of NO_2^- measured using Griess reagent could indirectly determine NO production by alveolar macrophages. Results demonstrated that the inhibition of NO was dose-dependent, irrespective to the drugs used (Figure 6B). The concentration of NO decreased from 38.3 ± 2.5 μM (untreated control) to 23.8 ± 4.7 μM, 12.5 ± 1.1 μM and 31.5 ± 3.1 μM when treated with 50 μM BD, RES and L-NAME, respectively. Resveratrol demonstrated the strongest activity, while NAME showed weak inhibitory effect across all concentrations assayed ($p < 0.05$). Only 25% inhibition of NO production could be observed even at high concentration (100 μM) of NAME used.

476 Comparatively, at the same concentration, RES and BD inhibited 75% and 60% of NO

477 production from alveolar macrophages, respectively (Figure 6B). The combination of

478 RES and BD was also studied to evaluate possible additive/synergistic inhibitory effects

479 of combination therapies towards NO productions in rat alveolar macrophage cells

480 (Figure 6C). Interestingly, the reduction of NO was more pronounced when RES and

481 BD were used in combination. The reduction was directly correlated to the increase of

482 RES in the formulation. The amount of NO produced showed the following trend: 23.8

483 $\pm$ 4.7 µM (0% RES or 100% BD) > 14.6 $\pm$ 0.7 µM (25% RES + 75% BD) > 12.5 $\pm$ 1.1

484 µM (100% RES or 0% BD) > 11.5 $\pm$ 1.1 µM (50% RES + 50% BD) > 9.9 $\pm$ 0.7 µM

485 (75% RES + 25% BD) (Figure 6C).

486

487 As mentioned, NO synthesis in alveolar macrophages is regulated by iNOS expression,

488 which in turn is controlled by NF-κB. Inhibiting high-output of NO by down-regulating

489 the iNOS expression at the transcription level, through inactivation of the NF-kB

490 signalling pathway, could be a treatment strategy (Cho, Koo et al. 2002; Li, Yan et al.

491 2002). In work by Li et al, the inhibition of NO and iNOS was achieved in LPS-

492 stimulated alveolar macrophages by pre-treating the cells with low concentrations of

493 BD (10^{-10} M) (Li, Yan et al. 2002). In contrast, higher concentrations of BD or RES

494 were needed in order to inhibit iNOS expression, as well as translocating NF-kB

495 compared to NO production in LPS-induced alveolar macrophages, consistent with our

496 results (Cho, Koo et al. 2002). In addition, RES was not effective to totally inhibit NO

497 productions once cells were stimulated with LPS. This suggests that both inhibition of

498 iNOS protein transcription and NF-kB inactivation is not the primary RES target

499 towards NO regulation. Cho et al hypothesized that RES interferes with LPS-induced

500   expression of genes responsible for NO expression, independent of the NF-kB

501   signalling pathway (Cho, Koo et al. 2002).

502

503

504   *3.3.3. Anti-inflammatory activity*

505   The anti-inflammatory properties of RES and BD have been widely documented.

506   Results showed that RES and BD alone inhibited cytokine expressions (IL-6 and TNF-

507   α) in LPS-stimulated alveolar macrophages as shown in Figure 7. These data are

508   consistent with other findings using either RES or BD to inhibit cytokine expressions in

509   different pulmonary cells line such as RAW 264.7, A549 and Calu-3 (Lilly, Nakamura

510   et al. 1997; Donnelly, Newton et al. 2004; Trotta, Lee et al. 2015). Based on these

511   results, RES is more effective than BD to reduce IL-6 and TNF-α ($p < 0.05$).

512   Approximately 60% and 20% of IL-6 was inhibited after 72 h of treatment with RES

513   and BD alone, respectively (Figure 7A). A similar inhibitory profile was observed for

514   TNF-α production. After 72 h treatment with RES and BD, only 45% and 32% of TNF-

515   α markers remained, respectively (Figure 7B). Our data also demonstrated that BD

516   preferentially inhibited the expression of TNF-α compared to IL-6 in LPS-stimulated

517   alveolar macrophage cells. This was supported by earlier findings whereby BD almost

518   completely inhibited TNF-α release, while only partially inhibited IL-6 expression from

519   macrophages (Linden and Brattsand 1994; Ek, Larsson et al. 1999). Our study clearly

520   demonstrated the time-dependent response for both RES and BD in inhibiting cytokines

521   (Figure 7). For BD, the percentages of TNF-α remained after 12 h, 24 h, 48 h and 72 h

522   were $83 \pm 4\%$, $76 \pm 3\%$, $70 \pm 2\%$, and $55 \pm 4\%$, respectively. As for RES, the amount of

523 IL-6 remained after 12 h, 24 h, 48 h and 72 h were $80.0 \pm 1.9\%$, $62.0 \pm 2.0\%$, $55.0 \pm$

524 2.2%, and $40.0 \pm 1.8\%$, respectively (Figure 7).

525

526 The effectiveness of corticosteroids in bacterial-induced COPD exacerbations remains

527 debatable. In a previous study, IL-8, MMP-9 and MCP-1 expressions from LPS-

528 stimulated alveolar macrophages were resistant to corticosteroids while IL-6 releases

529 were effectively inhibited (Knobloch, Hag et al. 2011). The authors also demonstrated

530 that the provision of RES alone reduced IL-8, MMP-9, IL-6 and MCP-1 expressions

531 down to baseline readings in alveolar macrophage isolated from COPD patients

532 (Knobloch, Hag et al. 2011). Therefore it was hypothesized that treatment of alveolar

533 macrophages with inhalable formulations containing BD and RES might enhance the

534 anti-inflammatory effect in COPD. As shown in Figure 7, cytokine releases from

535 alveolar macrophages were significantly inhibited with the presence of RES in the Co-

536 SD formulations. By increasing the ratio of RES from 0% to 75%, a reduction of IL-6

537 expression from 75% to 52% after 72h of treatment was observed. Similarly, significant

538 reductions of TNF-α production were observed by increasing RES (Figure 7). The exact

539 reason underlying stronger attenuation effect of RES towards cytokine compared to BD

540 is still unclear, but could be due to the antioxidant properties of RES in addition to its

541 anti-inflammatory effect.

542

543 The lack of corticosteroid efficacy could be the result of increased steroid-resistance in

544 pulmonary macrophages. The disparities in steroid sensitivity towards inflammatory

545 genes have been postulated to be attributable to impacts of corticosteroid-sensitive and

546 corticosteroid-resistant transcription mechanisms (Armstrong, Sargent et al. 2009,

547   Knobloch, Sibbing et al. 2010). In other words, the corticosteroids' efficacy towards

548   specific cytokine is highly dependent on the ratio of corticosteroid-sensitive and

549   corticosteroid-resistant signalling genes for transcriptions of cytokine proteins. For

550   instance, LPS-induced cytokine genes transcription in alveolar macrophages are

551   dependent on NF-κB signalling and mitogen-activated protein kinases (MAPK)/AP-1

552   pathway, whereby the former is sensitive to corticosteroids while the latter is non-

553   responsive to corticosteroids (Armstrong, Sargent et al. 2009). In contrast, RES is

554   known to be effective against both NF-κB and MAPK signalling (Cottart, Nivet-

555   Antoine et al. 2010), which could explain the higher efficacy of RES to reduce IL-6

556   from alveolar macrophages. Mechanistically, corticosteroid-resistance in COPD is also

557   augmented by reduced HDAC activities in alveolar macrophages caused by oxidative

558   and nitrosative stress (Barnes 2009). As RES scavenges ROS and RNS radicals readily,

559   RES could restore HDAC functions. This could also be a contributing factor for the

560   enhanced anti-inflammatory activity showed when combination of BD and RES was

561   administered.

562

4. Conclusions

564   In this study novel co-spray dried formulations of an anti-oxidant (RES) and anti-

565   inflammatory (BD) compounds were produced. The spray dried powders showed

566   appropriate morphologies and suitable aerosol properties for inhalation drug delivery. *In*

567   *vitro* studies showed that alveolar macrophages could tolerate RES and BD in the range

568   of concentrations investigated (0.612 to 50 μM). Moreover, RES and BD showed to

569   have anti-inflammatory activities due to their ability to reduce the levels of TNF-α and

570   IL-6. The data presented provide preliminary evidence that these compounds if

delivered in combination could be suitable for the treatment of chronic inflammatory lung diseases such asthma and COPD, where both inflammation and oxidative stress are present.

Declaration of interest

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Figure captions

Figure 1. Scanning electron microscopy images (SEM) of single and Co-SD formulations containing RES and BD at different ratios. (A) SD BD alone, (B) Co-SD containing 25% RES, (C) Co-SD containing 50% RES, (D) Co-SD containing 75% RES and (E) SD RES alone.

Figure 2. DSC thermograms of (A) raw BD, (B) SD BD alone, (C) Co-SD containing 25% RES, (D) Co-SD containing 50% RES, (E) Co-SD containing 75% RES ,(F) SD RES alone and (G) raw RES.

Figure 3. *In vitro* aerosol deposition of the single spray dried and Co-SD formulation using multi stage liquid impinger (MSLI) at flow rate of 60 l/min. Data represents mean ±SD (n=3). Black bar denotes resveratrol and white bar denotes budesonide.

Figure 4. *In vitro* aerosol deposition of the single spray dried and Co-SD formulation using multi stage liquid impinger (MSLI) at flow rate of 90l/min. Data represents mean ±SD (n=3). Black bar denotes resveratrol and white bar denotes budesonide.

Figure 5. Viabilities of alveolar macrophages cells evaluated using MTS cytotoxicity assay after 72 h of treatment with (A) single or (B) combination SD formulations. The percentages referred to the resveratrol concentration present in SD formulations. Data represents mean ±SD (n=3).

783 **Figure 6.** (A) The DPPH free radical scavenging activities. NO production in rat

784 alveolar macrophages (B) in the presence of lipoic acid, ascorbic acid, L-NAME, BD

785 and RES and (C) the effect of combination SD formulations. The percentages referred

786 to the resveratrol concentration present in SD formulations. Data represents mean ±SD

787 (n=3).

788

789 **Figure 7.** Cytokines expression in culture media of LPS pre-stimulated rat alveolar

790 macrophage cells after treatment with SD formulations at different time points. (A) IL-6

791 and (B) TNF-α. The percentages referred to the resveratrol concentration present in SD

792 formulations. Data represents mean ±SD (n=3).

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794